

Innervation of the Cerebral Superficial Arteries and the Parenchymal Small Vessels in *Elaphe quadrivirgata*

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ABSTRACT—The innervation of the cerebral arteries in the striped snake, *Elaphe quadrivirgata*, was studied by catecholamine fluorescence, acetylcholinesterase (AChE)-activation staining, and immunohistochemistry. Aminergic nerve fibers (Amn) showed dense meshworks of fine fibers not only in the cerebral carotid and basilar arteries but also in the cerebral parenchymal small vessels. Cholinergic nerve fibers (Chn) were distributed more densely than Amn in the cerebral carotid and basilar arteries, but were sparse in the cerebral parenchymal small vessels. The vasoactive intestinal polypeptide (VIP)-, substance P (SP)-, neurokinin A (NKA)- and calcitonin gene-related peptide (CGRP)-like immunoreactive (LI) nerve fibers were very densely distributed in the cerebral carotid and basilar arteries, and moderately distributed in the cerebral parenchymal small vessels. Neuropeptide Y (NPY)-LI nerve fibers were not detected in the superficial cerebral arteries, but they were distributed in the cerebral parenchymal small vessels. No differences of the distributions of Amn, Chn and peptide-LI nerve fibers were observed in the normal half side or in the undeveloped half of the cerebral carotid arteries. When examined by double staining, SP-, NKA- and CGRP-LI nerve fibers showed almost identical distribution patterns. Three different patterns existed concerning the relationship between Chn and VIP-LI nerve fibers: 1) Chn and VIP-LI nerve fibers coexisting, 2) Chn living alone, and 3) VIP-LI nerve fibers living alone.

INTRODUCTION

It has been thought that the cerebral blood vessels consist of the cerebral carotid and basilar systems which are controlled by aminergic nerve fibers (Amn) and cholinergic nerve fibers (Chn) whose neurotransmitters are noradrenaline and acetylcholine, respectively, with these neurotransmitters playing a main role in vasomotor modulation [1-9].

Recently, immunohistochemical developments have enabled the discovery of a number of peptide-like immunoreactive (LI) nerve fibers in the walls of cerebral blood vessels and, as a result, it appears that vasomotor modulation involves a complicated neuro-control mechanism [10-20]. However, since these findings have all been reported separately, a connection among the various nerves has not been elucidated. Moreover, these

studies were performed only for the superficial cerebral arteries in mammals and the examination of superficial cerebral arteries or parenchymal small vessels in submammalian animal species has rarely been performed.

Using immunohistochemical techniques, distributional patterns and the connection of Amn, Chn and peptide-LI nerve fibers, including substance P (SP)-, neurokinin A (NKA)-, calcitonin gene-related peptide (CGRP)-, vasoactive intestinal polypeptide (VIP)- and neuropeptide Y (NPY)-LI nerve fibers were examined in the superficial cerebral arteries and parenchymal small vessels of *Elaphe quadrivirgata*, which have a specific vascular system consisting of an undeveloped half side of the cerebral carotid artery.

MATERIALS AND METHODS

A group of 30 striped snakes, *E. quadrivirgata*, were used in this study. To detect Amn and Chn, the catecholamine fluorescence technique by

Falck-Hillarp [21] and the acetylcholinesterase (AChE)-activation staining technique by Karnovsky-Roots [22] were used. The detection of these nerves has been already reported in detail by Tagawa *et al.* [23].

In the immunohistochemical studies, each peptide-LI nerve fiber was detected using the peroxidase-antiperoxidase (PAP) technique [24] or the avidin-biotin peroxidase complex (ABC) technique [25], in the superficial cerebral arteries or the parenchymal small vessels, respectively.

After fixation by perfusion with cold Zamboni's solution [26], the cerebral parenchym was post-fixed in block form at 4°C for 24 hr with the same solution. The block was incubated in 0.1 M phosphate buffer (PB) containing 10% and 20% sucrose at 4°C for 48 hr each and mounted in gelatin. After re-fixation with Zamboni's solution at 4°C for 24 hr and incubating in 0.1 M PB containing 10% and 20% sucrose at 4°C for 48 hr each, the block was frozen with dry-ice isopentane and 15–20 μ m slices were reacted with the ABC reagent (Vector Inc., ABC KIT). The slices were treated with the first antibody (anti-SP, anti-NKA, anti-CGRP, anti-VIP and anti-NPY antibody) and the biotin conjugated second antibody at room temperature for 30 min each and the ABC reagent at room temperature for 60 min. They were then stained with peroxidase-substrate solution for 8 min, counter-stained with methyl-green. Then the parenchymal small vessels were observed under the microscope. Amn and Chn in the cerebral parenchym were observed in paraffin embedded slices using a catecholamine fluorescence techni-

que and in frozen slices using an AChE-activation staining technique, respectively.

After fixation by perfusion with cold Zamboni's solution, the whole-mount preparation of the superficial cerebral arteries, was re-fixed with the same solution at 4°C for 48 hr, and reacted for 1 hr with 0.1 M phosphate buffer saline (PBS) containing 1% H₂O₂ to abolish the non-specific peroxidase reaction. The PAP technique was performed while washing with 0.1 M PBS containing 0.3% Triton-X (PBST). After treatment with anti-SP, anti-NKA, anti-CGRP, anti-VIP and anti-NPY antibodies (at 4°C for 2 hr each) followed by washing with PBST, the slices were reacted with goat anti-rabbit IgG (Cappel PA, 1:200) at 4°C for 24 hr and again washed with PBST. They were then reacted with anti-rabbit PAP (Zymed, 1:200) at 4°C for 2 hr and stained with a solution of 3,3'-diaminobenzidine (DAB, Sigma). Lastly, the slices were dehydrated, mounted and observed under a microscope.

The coexistence of an autonomic neurotransmitter and a peptide, or a peptide and a peptide was examined using the double staining technique. In the double staining of VIP-LI nerve fibers and Chn, VIP-LI nerve fibers were stained by the PAP technique using 4-chloro-1-naphthol and next, Chn was identified with the AChE-activation staining technique. Double staining with SP-NKA or SP-CGRP was examined using a fluorescein labeled antibody technique [27]. The slices were treated with anti-rabbit NKA or anti-rabbit CGRP antibody at 4°C for 48 hr, followed by washing, and reacted with tetramethylrhodamine isothiocyanate

TABLE 1. Details of antibodies used

First antibody	Antibody code	Host	Dilution	Source	Reference
SP	FP03	Rabbit	1:5000	Dr. H. Kamiya Fukuoka Univ.	Takano <i>et al.</i> [33]
NKA	FK03	Rabbit	1:5000	Dr. H. Kamiya Fukuoka Univ.	Takano <i>et al.</i> [33]
CGRP	R.842701	Rabbit	1:1200	Milab Malmo, Sweden	Uddman <i>et al.</i> [34]
VIP	R.785202	Rabbit	1:1200	Milab Malmo, Sweden	Matsuyama <i>et al.</i> [13]
NPY	R.840403	Rabbit	1:1200	Milab Malmo, Sweden	Itakura <i>et al.</i> [29]
Mo-SP	B7A35	Rat	1:500	sera-lab England	Cuello <i>et al.</i> [35]

(TRITC)-labeled anti-rabbit IgG (SEIKAGAKU KOGYO Co., 1:200) at 38°C for 1 hr. Then, they were reacted with anti-rat monoclonal SP antibody at 4°C for 48 hr, washed and again reacted with fluorescein isothiocyanate (FITC)-labeled anti-rat IgG (ICN, 1:200) at 38°C for 2 hr. The slices were washed, mounted with glycerin-PBS (1:1) and observed under a fluorescence microscope.

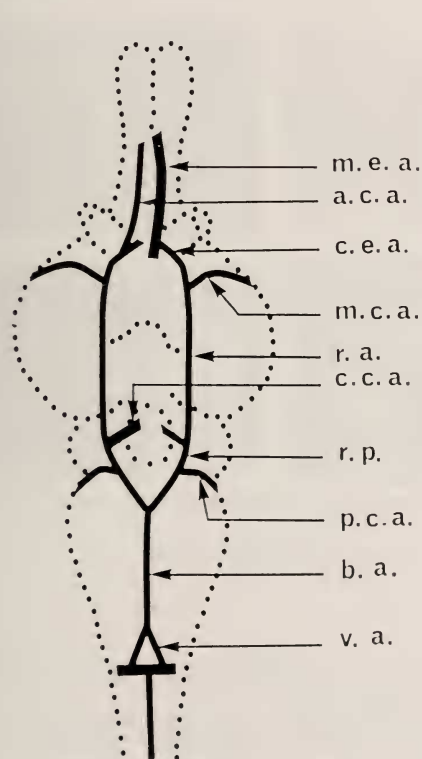
The names of the companies at which the first antibodies were prepared, and their dilution rates are shown in Table 1. In order to examine the specificities of the antibodies, absorption tests were performed.

RESULTS

A schematic illustration of the angio-architecture of the cerebral arteries in *E. quadrivirgata* is shown in Figure 1 and the distributional densities of Amn, Chn and peptide-LI nerve fibers in the superficial cerebral arteries and the parenchymal small vessels is shown in Table 2. Both the cerebral carotid and basilar arteries were found to be well innervated by Amn. Amn in the cerebral carotid and basilar arteries showed a dense distribution consisting of meshworks of fine fibers (Figs. 2, 3). These fine Amn were also evident in the cerebral parenchymal small vessels as well as in the superficial cerebral arteries (Fig. 4). In addition, the cerebral carotid and basilar arteries were well innervated by Chn with a very dense distribution of meshworks (Figs. 5, 6), although the distribution was sparse in the cerebral parenchymal small vessels (Fig. 7).

The distribution of VIP-LI nerve fibers was markedly dense, covering the cerebral carotid and basilar arteries and the cerebral parenchymal small vessels (Figs. 8–10). Although both SP- and NKA-LI nerve fibers were distributed rather densely in both the cerebral carotid and basilar arteries (Figs. 11, 12, 14, 15), their distributions in the cerebral parenchymal small vessels were far more dense as compared with the cerebral carotid and basilar arteries (Figs. 13, 16). CGRP-LI nerve fibers revealed a very dense distribution over the cerebral carotid arteries (Fig. 18), but their distributions in the basilar arteries and the parenchymal small vessels were only moderately dense (Figs. 17, 19). Although NPY-LI nerve fibers could not be observed in either the cerebral carotid or basilar system, they were found with NPY-positive nerve cells in the cerebral parenchyma. No differences were found among the distributional densities of Amn, Chn or peptide-LI nerve fibers in the cerebral carotid arteries in the normal half side or in the undeveloped half.

A similar distributional pattern was shown among SP- and NKA-LI or SP- and CGRP-LI nerve fibers when examined by double staining (Figs. 20, 21). However, VIP-LI nerve fibers and Chn were found in three patterns: 1) VIP-LI nerve fibers and Chn existing together, 2) VIP-LI fibers



Elaphe quadrivirgata atra

FIG. 1. Diagram of the arterial supply to the ventral surface of the brain in the *Elaphe quadrivirgata*. m.e.a. ethmoidal artery; a.c.a. anterior cerebral artery; c.e.a. cerebro-ethmoidal artery; m.c.a. middle cerebral artery; r.a. anterior ramus; c.c.a. cerebral carotid artery; r.p. posterior ramus; p.c.a. posterior cerebral artery; b.a. basilar artery; v.a. vertebral artery.

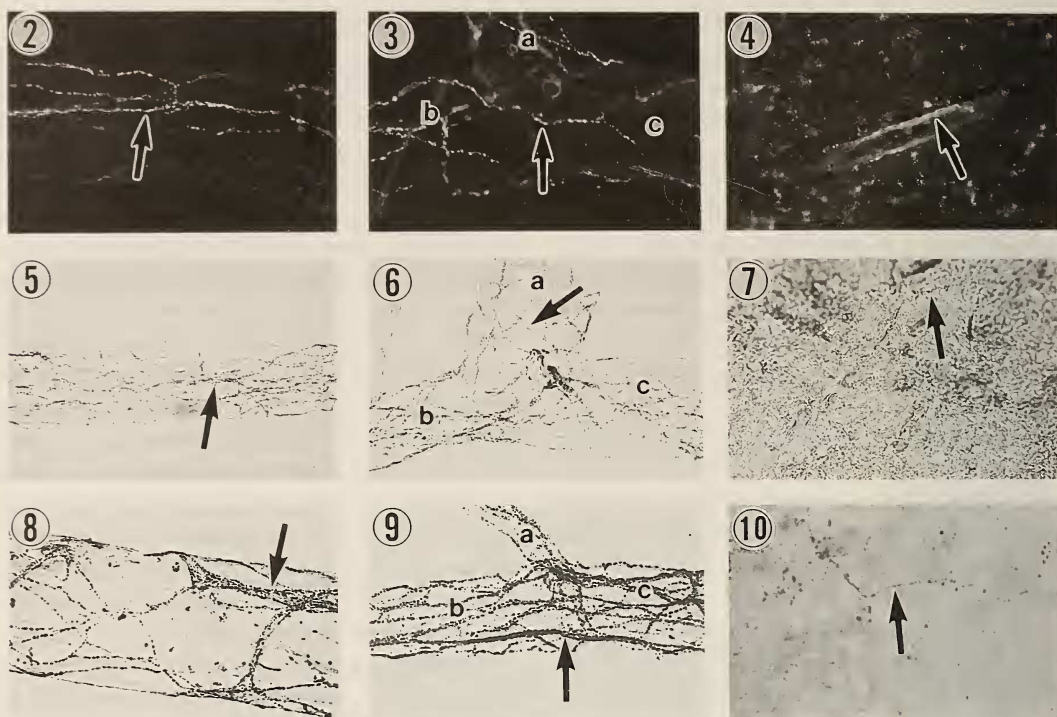


FIG. 2. Aminergic innervation of the cerebral artery. Arrow indicates Amn. $\times 100$.

FIG. 3. Amn (arrow) of the cerebral carotid artery and its branch. a. cerebral carotid artery; b. posterior ramus; c. anterior ramus $\times 100$.

FIG. 4. Amn (arrow) which distributed in the small vessel of cerebral parenchym. $\times 250$.

FIG. 5. Chn (arrow) of the basilar artery. $\times 100$.

FIG. 6. Chn (arrow) of the cerebral carotid artery and its branch. a. cerebral carotid artery; b. posterior ramus; c. anterior ramus $\times 100$.

FIG. 7. Chn (arrow) in the cerebral parenchymal small vessel. $\times 100$.

FIG. 8. VIP-LI nerve fiber (arrow) of the basilar artery. $\times 100$.

FIG. 9. VIP-LI nerve fiber (arrow) of the cerebral carotid artery and its branch. a. cerebral carotid artery; b. posterior ramus; c. anterior ramus $\times 100$.

FIG. 10. VIP-LI nerve fiber (arrow) which distributed in the cerebral parenchym. $\times 160$.

TABLE 2. Density of Amn, Chn and peptide-like immunoreactive (LI) fibers of cerebral arteries and intracerebral parenchymal small vessels in *Elaphe quadrivirgata*

	Amn	Chn	SP-LI fiber	NKA-LI fiber	CGRP-LI fiber	VIP-LI fiber	NPY-LI fiber
cerebral carotid arteries	++	+++	+	+	+++	+++	—
basilar arteries	++	+++	+	+	+	+++	—
intracerebral parenchymal small vessels	++	±	+++	+++	+	+++	+

The relative number of nerves was graded arbitrarily; — no nerve was observed

±. few nerves

+ moderate number of nerves |

++ rich supply of nerves |

+++ very rich supply of nerves |

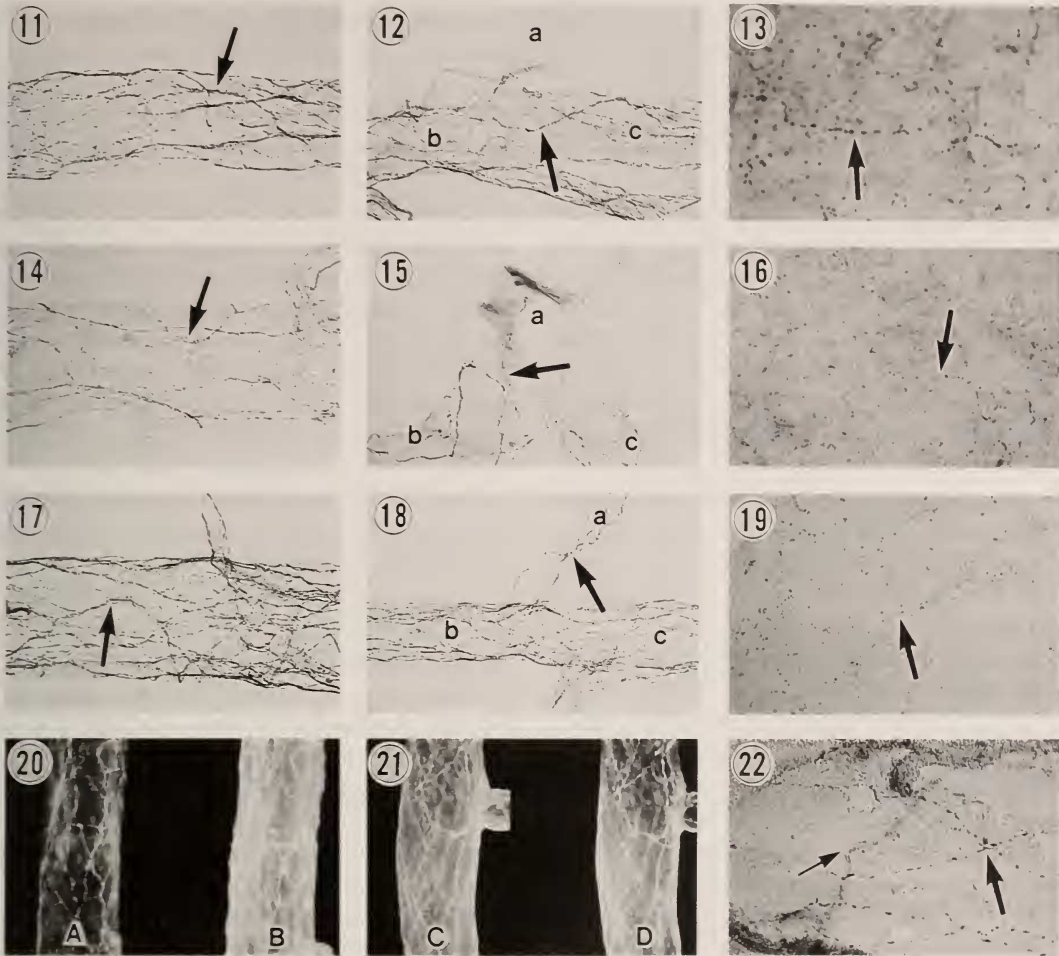


FIG. 11. SP-LI nerve fiber (arrow) of the basilar artery. $\times 100$.

FIG. 12. SP-LI nerve fiber (arrow) of the cerebral carotid artery and its branch. a. cerebral carotid artery; b. posterior ramus; c. anterior ramus $\times 100$.

FIG. 13. SP-LI nerve fiber (arrow) which distributed in the cerebral parenchym. $\times 160$.

FIG. 14. NKA-LI nerve fiber (arrow) of the basilar artery. $\times 100$.

FIG. 15. NKA-LI nerve fiber (arrow) of the cerebral carotid artery and its branch. a. cerebral carotid artery; b. posterior ramus; c. anterior ramus $\times 100$.

FIG. 16. NKA-LI nerve fiber (arrow) which distributed in the cerebral parenchym. $\times 160$.

FIG. 17. CGRP-LI nerve fiber (arrow) of the basilar artery. $\times 100$.

FIG. 18. CGRP-LI nerve fiber (arrow) of the cerebral carotid artery and its branch. a. cerebral carotid artery; b. posterior ramus; c. anterior ramus $\times 100$.

FIG. 19. CGRP-LI nerve fiber (arrow) which distributed in the cerebral parenchym. $\times 100$.

FIG. 20. SP-LI (A) and NKA-LI (B) nerve fibers of the posterior cerebral artery. $\times 100$.

FIG. 21. CGRP-LI (C) and SP-LI (D) nerve fibers of the posterior cerebral artery. $\times 100$.

FIG. 22. Chn and VIP-LI nerve fiber of the posterior cerebral artery. Large arrow indicates VIP-LI nerve fiber and small arrow Chn. $\times 100$.

observed alone and 3) Chn observed alone (Fig. 22).

DISCUSSION

Nearly comparable amounts of Amn and Chn in the superficial cerebral arteries were reported in

rats [8] and larger amounts of Amn as compared with Chn were reported in domestic fowls [23] and tortoises [6], while, in bullfrog [7] or lamprey [28], Amn or serotonergic nerves alone were found. The distributional densities of both Amn and Chn were higher in the cerebral carotid system than in the basilar system in rats [8], domestic fowls [23] and tortoises [6], whereas, in bullfrogs [7], Amn alone was found, with larger amounts in the cerebral carotid system than in the basilar system.

However, in *E. quadrivirgata*, the distributional density of Amn was nearly equal in both the cerebral carotid and basilar systems and a dense meshworks of fine Amn was found in all the areas of the cerebral carotid and basilar systems. Chn was nearly equally and densely distributed in both arterial systems. Chn density was higher than that of Amn in both systems. These findings suggest that Amn and Chn possessed different densities at each site depending upon the species.

Among the peptide-LI nerve fibers, SP-, NKA-, CGRP- and VIP-LI nerve fibers were each found to possess a similar density between the cerebral carotid and basilar systems. The distributions of CGRP- and VIP-LI nerve fibers were more dense compared with those of SP- and NKA-LI nerve fibers in both the system. These findings are not coincidental with the results previously presented [11, 17, 29, 30], in which the distributions of peptide-LI nerve fibers were higher in the cerebral carotid system than in the basilar system. However, according to a study in our laboratory on hamsters, a difference in the distributions of SP- and CGRP-LI nerve fibers was not observed between these two arterial systems, although VIP-LI nerve fibers alone possessed a higher density in the cerebral carotid system. These findings suggest that the distributional mode and density of peptide-LI nerve fibers in the cerebral arteries differ considerably with each order or species.

The distribution in the cerebral parenchymal small vessels of each kind of nerve varied considerably from those in the cerebral carotid and basilar systems; the SP-, NKA- and VIP-LI nerve fibers were distributed very densely while the Chn alone were very sparse. Survival of the reptiles was rare and like in the case of birds after anesthesia for experimental purposes, the origin and fibrous pro-

longation of superficial and deep vessels was not elucidated. Nevertheless, it seems that the existence of NPY-positive nerve cells and NPY-LI nerve fibers in the cerebral parenchyma suggests an objective for the study of the origin of peptide-LI nerve fibers in the future.

In mammals the coexistence of Amn, Chn and peptide-LI nerve fibres or that of differing peptidergic nerve fibers was reported. In the cerebral arteries of *E. quadrivirgata*, the mode and the density of distribution were mutually equal between SP- and NKA-LI nerve fibers and between SP- and CGRP-LI nerve fibers and accordingly, the coexistence of SP, NKA and CGRP in the same fiber was suggested. The coexistence of SP and CGRP in the cerebral arteries or trigeminal ganglion was reported by Hanks *et al.* [18] in cats and Matsuyama *et al.* [17] in rats and guinea pigs. Although Lundberg *et al.* [31] reported the coexistence of VIP-LI nerve fibers and Chn in cat submaxillary glands and Kobayashi *et al.* [11] as well as Hara *et al.* [20] reported this in rat cerebral arteries, the distributional mode of VIP-LI nerve fibers and Chn in the superficial cerebral arteries of *E. quadrivirgata* was mutually different in spite of their similar distributional density. Three patterns were observed: 1) the coexistence of VIP-LI nerve fibers and Chn, 2) VIP-LI nerve fibers alone and 3) Chn alone. This is coincidental with a connection between VIP-LI nerve fibers and Chn in the cerebral arteries of hamsters [unpublished] and *Leiothrix lutea* [unpublished] examined in our laboratory.

Amn [2, 3] and NPY [14, 32] have a vasoconstrictive action and Chn [4, 9] and VIP [10, 14] have a vasodilative action. Although the direct action of SP, NKA and CGRP for the vascular smooth muscle is vasoconstrictive, it is said to be vasodilative when vascular endothelia exist together. Either way, elucidation of the origin and the fibrous prolongation as well as the function of each kind of nerve was not possible because an anesthetic technique for *E. quadrivirgata* has not been established and their survival after biopsy is impossible. In the future, an anesthetic technique should be developed and various morphologic, pharmacologic and physiologic studies of these remaining questions should be undertaken.

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